Title

Radiation dose-escalation and dose-fractionation modulate the immune microenvironment, cancer stem cells and vasculature in experimental high-grade gliomas

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Running title

Radiotherapy modulates brain tumor immunity, stem cells and vasculature

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Conflict of interest

The Authors declare no conflict of interest.

Authorship

Conception of the study: MR, AC, MvR, RG

Design of the experiments: MR, AC

Radiotherapy: MR, RW, ES, UH

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Analysis and interpretation of the results: MR, JC, MvR, AC, RG

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Abstract

Introduction. In the context of radioimmunotherapy combinations for high-grade gliomas (HGGs), very few evidences are~~is~~ available concerning the optimal radiotherapy (RT) dosage and fractionation.

Methods. Neurospheres (NS) CT-2A HGG-bearing C57BL/6 mice were treated with stereotactic RT. For dose-escalation experiments, mice received 2, 4 or 8 Gy as single administrations. For dose-fractionation experiments, mice received 4 Gy as a single fraction or multiple (1.33x3 Gy) fractions. The impact of the RT schedule on murine survival and tumor immunity was evaluated. Modifications of glioma stem cells (GSCs), tumor vasculature and tumor cell replication were also assessed.

Results. RT dose-escalation was associated with an improved immune profile, with higher $CD8^+$ T cells and $CD8^+$ T cells / regulatory T cells (Tregs) ratio (p=0.0003 and p=0.0022, respectively) and lower total tumor associated microglia/macrophages (TAMs), M2 TAMs and monocytic myeloid derived suppressor cells (mMDSCs) (p=0.0011, p=0.0024 and p=0.0001, respectively). The progressive increase of RT dosages prolonged survival (p<0.0001) and reduced tumor vasculature (p=0.069), tumor cell proliferation (p<0.0001) and the amount of GSCs (p=0.0132 or lower). Compared to the unfractionated regimen, RT dose-fractionation negatively affected tumor immunity by inducing higher total TAMs, M2 TAMs and mMDSCs (p=0.0051, p=0.0036 and p=0.0436, respectively). Fractionation also induced a shorter survival (p=0.0078), a higher amount of GSCs (p=0.0015 or lower) and a higher degree of tumor cell proliferation (p=0.0003).

Conclusions. This study demonstrates that RT dosage and fractionation significantly influence survival, tumor immunity and GSCs in experimental HGGs. These findings should be taken into account when aiming at designing more synergistic and effective radio-immunotherapy combinations.

Key words

Glioblastoma

High-grade glioma

Radiotherapy

Dose-escalation

Dose-fractionation

Tumor immunity

Glioma stem cells

Introduction

The standard treatment for glioblastoma (GBM) patients includes surgical removal followed by fractioned radiotherapy (RT, 60Gy in 2Gy fractions) and Temozolomide (TMZ).[1,2] However, despite such multimodal therapy, the prognosis of GBM patients remains extremely poor with a median survival of approximately 15 months.[3] In recent years, the evidence that RT is capable to start a potent anti-tumor immune reaction led to many clinical trials assessing the efficacy of radio-immunotherapy combinations for GBM.[4,5]In most of them, the standard conventionally-fractioned RT regimen was used; however, it has to be taken into account that such RT schedule has been established long before the development of immunotherapy and it is therefore not optimized for this type of combination.[5,6] To date, very limited evidence is available concerning ~~which~~ what could be the optimal RT dosage and fractionation to be used in radio-immunotherapy combinations for GBM.[7]

In this study, we aimed at filling this knowledge gap by analyzing the effects of RT dose-escalation and dose-fractionation on the tumor microenvironment (TME) of experimental high-grade gliomas (HGG).

Methods

All animal experiments were approved by the Animal Ethics Committee of the Katholieke Universiteit Leuven (project 089/2015) which follows the European Directive 2010/63/EU. Below is a summary of the Methods, more details are available as Supplementary material 1. The datasets produced, used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Brain tumor model and experimental design. An orthotopic, immunocompetent HGG model recently developed by our group was used for this study[8] .~~8~~ This model is based on the implantation of CT-2A neurospheres (NS/CT-2A) and it is specifically designed to address the tumor-induced immune suppression. CT-2A cells were provided by Prof. Thomas Seyfried (Boston College, Boston, MA).[9] Tumor engraftment was confirmed via brain magnetic resonance imaging (MRI). In the dose escalation study, the effects of a single RT dose of 2, 4 or 8 Gy was evaluated. In the dose-fractionation study, the effects of a cumulative RT dose of 4 Gy administered either in one single or in three equal fractions (of 1.33 Gy each) was tested.

Magnetic resonance imaging (MRI). Brain MRI was performed using a 7 tesla~~T~~ ~~Biospec~~ horizontal small animal MR system (Biospec 70/30, Bruker BioSpin). During the MRI scans, animals were anesthetized with isoflurane (2% in pure oxygen) and placed in a water-heated dedicated MRI compatible mouse bed. All data were acquired through a quadrature volume coil with an internal diameter of 86mm for radiofrequency transmission and a decoupled mouse brain surface coil for signal reception (bruker Biospin).

Rapid acquisition relaxation enhancement (RARE) T2-weighted (T2w) images were used to verify tumor engraftment and to calculate tumor volumes, as previously described.[8]

Radiotherapy. Stereotactic external beam image-guided arc irradiation was performed using a Small Animal Radiation Research Platform (SARRP, Xstrahl Life Sciences). Each irradiated mouse received a single dose of 4 Gy. The treatment plan was designed in order to deliver the full radiation dose to any visible tumor and to spare the largest part of the surrounding healthy brain. Details of the radiotherapy workflow are available as Supplementary material 2.

Brain tumor infiltrating immune cells. Mononuclear cells were isolated from the brain of tumor-bearing mice and stained for tumor-associated microglia/macrophages (TAMs), monocytic and granulocytic myeloid-derived suppressor cells (mMDSCs and gMDSCs) and T cells as previously described.[8]

Immunohistochemistry (IHC). IHC was used to evaluate GSCs, tumor vasculature and tumor cell proliferation. The following primary antibodies directed against the respective proteins were used to evaluate GSCs: anti-cluster of differentiation (CD) 133, anti-Nestin and anti-octamer-binding transcription factor (Oct) 4. Blood vessels were stained with anti-CD31 antibody while cell proliferation was evaluated by means of anti phosphohistone (PH) 3. IHC images were acquired with a Zeiss AxioScan Z1 using an x20 objective and the software ZEN 2 (Zeiss). The software QuPath was used to assess CD133, Nestin, Oct4 and PH3 stainings whereas the software ImageJ (http://rsb.info.nih.gov/ij) was used to assess CD31 stainings.[10]

Statistical analysis. Statistical analysis was performed using Prism v.8.0 (Graphpad Software) and a 2-tailed p-value <0.05 was considered significant. Survival data were compared using log-rank (Mantel-Cox) test and adjustment for multiple survival comparisons was performed with Benjamini-Hochberg method. In the dose-escalation experiments, flow-cytometry and IHC data underwent a primary and a secondary analysis. For the primary analysis, the RT dosage was considered as a continuous variable and its correlation with flow cytometry or IHC data was tested via Pearson's r and linear regression. For the secondary analysis, the RT dosage was considered as a discrete variable and IHC or flow-cytometry data

were compared with ANOVA (for normally distributed data) or non-parametric Kruskal-Wallis test (for non-normally distributed data). In dose-fractionation experiments, the RT dosage was considered as discrete variable and IHC or flow-cytometry data were compared as for the secondary analysis of dose-escalation experiments.

Results

Higher radiation dosages are associated with prolonged survival, more favorable immune profile and reduced GSCs, tumor vasculature and tumor cell replication

In the dose-escalation study, mice were treated with 2, 4 or 8 Gy RT administered in one single fraction. Control mice were left untreated. As depicted in Figure 1, treated mice survived significantly longer than untreated controls and the RT dose-escalation induced a progressive increase of survival (Log-rank $p<0.0001$). Median survival was 26 days for control mice, 31 days for mice receiving 2 Gy ($p_{adj}=0.0075$ versus controls) and 36 days for mice receiving 4Gy ($p_{adj}=0.0239$ versus 2Gy). The median survival was not reached for mice receiving 8 Gy, since in this group we observed 75% long-term survivors ($p_{adj}=0.005$ versus 4 Gy).

The primary and the secondary analysis consistently showed that RT dose-escalation positively modulated tumor immunity by inducing a progressive increase of anti-tumor immune cells and a progressive decrease of immune suppressive cells in the context of both the adaptive and innate system. In the primary analysis (Figure 2), increasing RT dosages were associated with an increase in $CD8^+$ T cells ($r=0.7234$, $p=0.0003$) and $CD8^+$ T cells / regulatory T cells (Tregs) ratio ($r=0.2837$, $p=0.0022$) whereas a decrease in total TAMs ($r=-0.4537$, $p=0.0011$), immunosuppressive M2 TAMs ($r=-0.6389$, $p=0.0024$) and monocytic MDSCs (mMDSCs; $r=-0.9032$, $p<0.0001$) was observed. No significant association was found between the radiation dosage and $CD4^+$ T cells, Tregs and granulocytic MDSCs (gMDSCs; $p=0.1008$, $p=0.1269$ and $p=0.9844$ respectively). The secondary analysis provided similar results; however, the differences in TAMs did not reach statistical significance because of the relatively large standard deviation (Supplementary Material 2).

The IHC assessment of GSCs, tumor vasculature and tumor cell replication was performed at the same time point of the immune analysis. As shown in Figure 3, the primary analysis demonstrated the positive impact of dose escalation on GSCs since the increase of RT dosages was negatively associated with all the three GSCs markers analyzed: Nestin ($r=-0.6193$, $p<0.0001$), CD133 ($r=-0.3554$,

p=0.0132) and Oct4 (r=-0.4563, p=0.0011). A significant decrease of the proportion of replicating tumor cells (PH3 staining) and a trend towards a reduction of CD31+ tumor vessels were also observed (r=-0.8641, p=0.0001 and r=-0.2648, p=0.0690 respectively). The results of the secondary analysis confirmed those of the primary; however, statistical significance was not reached for CD133+ GSCs due to the relatively large standard deviation (Supplementary Material 2). Representative IHC images of Nestin, CD133, Oct4, CD31 and PH3 stainings in the dose-escalation study are shown in Supplementary Material 3.

Fractionated RT is associated with shorter survival and less favorable immune profile, and it has a reduced efficacy in the context of GSCs and tumor cells replication

In the dose-fractionation study, all treated mice received the same cumulative RT dose of 4 Gy which was either given in one single administration or divided in three equal fractions of 1.33 Gy. A control group of untreated mice was included only in the survival analysis. As shown in Figure 4, RT dose-fractionation had a negative impact on mice survival. Unfractioned RT significantly prolonged survival compared to controls (36 vs 27 days, p_{adj}=0.0228). Conversely, such survival advantage was completely lost in case of fractioned RT, since mice undergoing this treatment survived significantly shorter than those undergoing the unfractioned regimen (30 vs 36 days, p_{adj}=0.0117). Furthermore, the survival of mice treated with fractioned RT was not different from that of the controls (30 vs 27 days, p_{adj}=0.31).

As shown in Figure 5, RT dose-fractionation had a negative impact on the innate immunity, inducing higher total TAMs (p=0.0051), M2 TAMs (p=0.0036) and mMDSCs (p=0.0436) compared to the non-fractioned regimen. Of note, mice treated with fractioned RT exhibited significantly lower gMDSC (p=0.0450); however, this cell type was approximately ten times less abundant than its monocytic counterpart. Conversely, no significant differences in T cells populations were observed. $CD8^+$ T cells and $CD4^+$ T cells were comparable in the fractioned and non-fractioned regimens (p=0.6052 and p=0.9604, respectively). We found a trend towards lower Tregs in the fractioned RT group (p=0.0639),

however the $CD8^+$ T cells / Tregs ratio, which provides an indication about the balance between immune activation and immune suppression, was comparable in the two treatment groups (p=0.6031). Unfractioned RT was also more effective in eliminating GSCs compared to the fractioned schedule (Figure 6). In particular, tumors irradiated with one single dose of 4 Gy significantly showed lower $Nestin^+$ and $Oct4^+$ GSCs compared to those irradiated with three doses of 1.33 Gy each (p=0.0015 and p=0.001 respectively). No differences were observed in the context of $CD133^+$ GSCs (p=0.189). The unfractioned regimen was more effective in reducing tumor cells proliferation (p=0.0003) while tumor vasculature was similar in the two treatment groups (p=0.6707). Representative IHC images of Nestin, CD133, Oct4, CD31 and PH3 stainings in the dose-fractionation study are shown in Supplementary Material 3.

Discussion

In recent years, many preclinical studies demonstrated that RT could potentiate the effects of cancer immunotherapy.[11–13] Therefore, a number of clinical trials evaluating the efficacy of radio-immunotherapy combinations in patients, including those diagnosed with HGG, has been started.[14] However, two important issues prevent from directly translating into HGG patients the results obtained in radio-immunotherapeutic animal studies. First, most preclinical evidence concerning the immune effects of RT in cancer has not been obtained in HGG models: this could have relevant consequences given the peculiar aspects of the brain's immune system.[15] Second, there is an important methodological difference between the available HGG preclinical studies and the ongoing clinical trials: rodents are usually treated with one single RT administration, while HGG patients are being treated (with few exceptions) with the usual conventionally-fractioned RT schedule (30 x 2 Gy).[16] Well-established evidence proves that RT can potentiate the anti-tumor immune reaction; nevertheless, it has also been shown that RT can be immune suppressive.[4–6,12,17,18] In this balance between immune activation and suppression, RT dosage and fractionation play a critical role.[12,16,19,20] Interestingly, this differential immune modulation might become apparent only when RT is combined with immunotherapy: in the same breast carcinoma models, two different RT regimens had equal effects when used alone but showed different efficacy in combination with checkpoint blockade.[21] All this demonstrates that the optimal RT schedule for radio-immunotherapy combinations still has to be defined, in particular for HGGs. The present study was specifically aimed at addressing this knowledge gap.[7,14,19]

This study was performed using the NS/CT-2A model, a fully immunocompetent orthotopic HGG model recently developed by our group.[8] When RT was administered in one single session, dose escalation was positively associated with survival and the highest RT dosage (8 Gy) was also capable to cure 75% of the animals. This can be explained by immune and non-immune phenomena. In term of immune effects, we demonstrated not only that single-fraction RT can positively modulate HGG

immunity (confirming previous literature)[14] but also that such modulation is dose dependent and involves both the adaptive and innate immune compartments. In terms of non-immune effects, we showed that RT dose escalation is negatively associated with the expression of PH3, a proliferation marker which has been reported to be potentially more accurate than Ki67[22]. In line with the traditional radio-biological concepts, this is explained by the capacity of ionizing radiations to induce DNA damage which is followed by cell-cycle arrest and apoptosis.[4] To dissect the immunological role of RT dose-fractionation, we treated NS/CT-2A tumor-bearing mice with a cumulative dosage of 4 Gy given either in one single administration or fractioned in three equal doses. Such dosage was chosen since it provided the strongest survival benefit without generating any long-term survivor: this is in line to what is observed in HGG patients where RT alone does not allow for a definitive cure.[2] Interestingly, mice receiving the single irradiation survived significantly longer than mice undergoing the fractioned regimen, which in turn had a survival comparable to controls. We did not find significant differences in term of adaptive immunity between these two RT schedules. However, significantly higher innate immune-suppressive cells (total TAMs, M2 TAMs and mMDSCs) were found following the fractionated regimen. In addition, tumors treated with single-dose RT showed a significantly lower cell replication. Taken together, our results show the positive impact of RT dose-escalation and the negative impact of RT dose-fractionation on HGG immunity.

Another area of active research concerns GSCs, a subpopulation of HGG cells which has been shown to contribute to the HGG radio-resistance due to their increased capacity of DNA repair.[23–28] In the present study, we investigated the impact of RT dose escalation and fractionation on GSCs by evaluating the expression of three GSCs markers (Nestin, CD133 and Oct4), two of which (Nestin and CD133) have been recently proven to be associated with a significantly worse prognosis in a recent meta-analysis.[29] In our experiments, RT dose-escalation reduced the expression of all the three GSC markers analyzed. Conversely, mice receiving fractioned RT showed significantly higher amounts of $Nestin^+$ and $Oct4^+$ GSCs compared to mice receiving non-fractionated RT. These results indicate that RT

dosage and fractionation influences the GSC niche in HGG. Similarly to tumor immunity, also in this case the dose-fractionation had a detrimental impact whereas the dose-escalation had a positive impact.

This study suffers some limitations. First, the experiment were performed in one single tumor model (NS/CT-2A). However, the NS/CT-2A was specifically designed to assess immune suppression, GSCs and vascular proliferation; therefore, it is particularly suitable for this type of studies.[8] Second, tumor infiltrating immune cells were analyzed only with flow-cytometry which does not provide information concerning the spatial location of such cells. However, this technique allows for a detailed characterization of several subtypes of immune cells which would have not been ~~not~~ possible with other techniques such as IHC (for example MDSC). Third, the RT dosages used in this study do not correspond exactly to what is used in the clinic. This was due to the higher responsiveness to ionizing radiations of NS/CT-2A tumors compared to human gliomas. However, we explored a broad range of dosages from 2 Gy (which corresponds to the single fractionations given to patients in the standard fractionated regimen) until 8 Gy (which induced 75% of long term survivors, an effect much stronger than what is seen in patients). Furthermore, for the dose-fractionation experiments, we used cumulative dosages (4Gy) which was able to prolong survival without inducing long-term survivors: this closely mimics what observed in GBM patients.

Conclusions

Many clinical trials combining RT and immunotherapy for the treatment of malignant gliomas are currently ongoing. Nevertheless, very limited evidence is available concerning the optimal RT schedule to be used in such radio-immunotherapy combinations. In this study, we demonstrated for the first time that dose escalation and fractionation have a strong impact on the immune microenvironment of experimental malignant gliomas. In particular, RT dose-escalation is associated with a more favorable immune profile whereas RT dose-fractionation is immunologically detrimental. Similarly, fractioned RT shows a lower efficacy in eliminating GCSs and reducing tumor cell proliferation compared to the unfractionated treatment. These results might have relevant implications for future clinical trials, given that most HGG patients receiving radio-immunotherapy combinations are treated with a fractionated regimen.

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Figure legends

Figure 1. **Survival of mice following RT dose-escalation**. NS/CT-2A HGG-bearing mice were treated with 2, 4 or 8 Gy given in one single administration while control mice were left untreated. All treated mice survived significantly longer (p_{adj}=0.0075, padj=0.00025 and p=0.005 for 2, 4 and 8 Gy, respectively) than control mice and the increase of the radiation dose was associated with increased survival. Survivals were compared by using the log-rank (Mantel-Cox) test and adjustment for multiple survival comparisons was performed with Benjamini-Hochberg method.

Figure 2. **Modifications of the tumor-immune microenvironment induced by RT dose-escalation**. NS/CT-2A HGG-bearing mice were treated with 2, 4 or 8 Gy given in one single administration while control mice were left untreated. Tumor-infiltrating immune cells were analyzed with flow-cytometry seven days after the treatment. In the adaptive immune compartment (panel A, B, C and D), the RT dose-escalation was positively correlated with $CD8^+$ T cells and the $CD8^+$ T cells / Tregs ratio. No correlation were seen between the radiation dose and $CD4^+$ T cells or Tregs. In the innate immune compartment (panel E, F, G and H), the RT dose-escalation was negatively correlated with total TAMs, M2 TAMs and mMDSCs. No correlation was found between the radiation dose and gMDSCs. Immune cells are expressed as percentage of $CD45^+$ cells. CD: cluster of differentiation; Tregs: regulatory T cells; TAMs: tumor associated macrophages/microglia; m and gMDSCs: monocytic and granulocytic myeloid-derived suppressor cells

Figure 3. **Impact of RT dose-escalation on GSCs, tumor vasculature and tumor cell replication**. NS/CT-2A HGG-bearing mice were treated with 2, 4 or 8 Gy given in one single administration while control mice were left untreated. Seven days after treatment, brains were collected and stained for immunohistochemistry. RT dose-escalation was negatively correlated with the expression of the GSC markers Nestin (A), CD133 (B) and Oct4(C). The increase of the radiation dosage induced a tendency

towards a reduction of the tumor vasculature (D) and was negatively correlated with the proliferation marker PH3. HGG: high-grade gliomas; CD: cluster of differentiation; Oct4: octamer-binding transcription factor 4; PH3: phosphohistone H3.

Figure 4. **Survival of mice following RT dose-fractionation**. NS/CT-2A HGG-bearing mice were treated with a cumulative dosage of 4 Gy, which was delivered either in one single administration or in three equal fractions of 1.33 Gy each. Control mice were left untreated. Mice treated with the unfractioned regimen survived significantly longer than mice receiving fractionated RT and controls. The survival of mice treated with the fractionated regimen did not differ from controls. Survivals were compared by using the log-rank (Mantel-Cox) test and adjustment for multiple survival comparisons was performed with Benjamini-Hochberg method.

Figure 5. **Modifications of the tumor-immune microenvironment induced by RT dose-fractionation**. NS/CT-2A HGG-bearing mice were treated with a cumulative dosage of 4 Gy, which were delivered either in one single administration or in three equal fractions of 1.33 Gy each. Tumor-infiltrating immune cells were analyzed with flow-cytometry seven days after the start of the treatment. No significant differences were found in the analysis of the adaptive compartment of the tumor-immune micro environment (panel A, B, C and D). Conversely, differences were seen in the innate immune system where unfractioned RT induced a better immune profile with significantly lower total TAMs, M2 TAMs and mMDSCs (panel E, F and G, respectively). On the contrary, gMDSCs showed the opposite behavior and where significantly higher in the after unfractioned RT. Immune cells are expressed as percentage of $CD45^+$ cells. CD: cluster of differentiation; Tregs: regulatory T cells; TAMs: tumor associated macrophages/microglia; m and gMDSCs: monocytic and granulocytic myeloid-derived suppressor cells

Figure 6. Impact of RT dose-fractionation on GSCs, tumor vasculature and tumor cell replication. NS/CT-2A HGG-bearing mice were treated with a cumulative dosage of 4 Gy, which were delivered either in

one single administration or in three equal fractions of 1.33 Gy each. Seven days after the start of treatment, brains were collected and stained for IHC. $Nestin^+$ (A) and $Oct4^+$ GSCs were significantly lower after the unfractionated RT compared to fractionated RT, while no significant differences were found in the expression of CD133 (C). CD: cluster of differentiation; Oct4: octamer-binding transcription factor 4; PH3: phosphohistone H3.